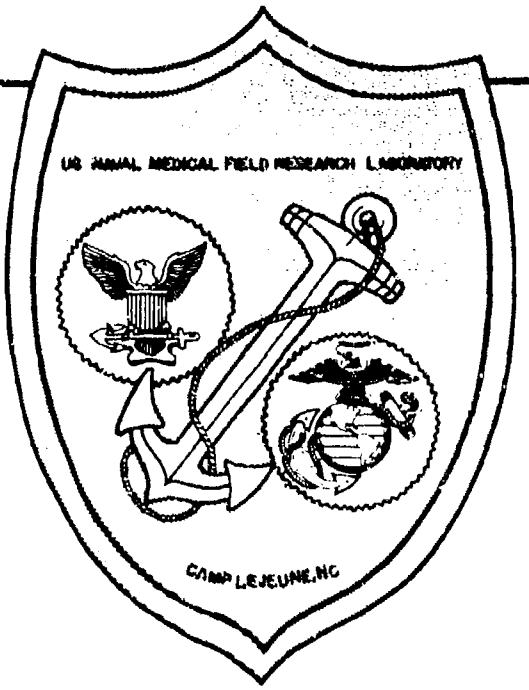

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THE RAPID DIAGNOSIS OF ACUTE RESPIRATORY DISEASE DUE TO ADENOVIRUS: Preparation and Standardization of Fluorescent Antibody Reagents

by

LT David P. McCormick, MC, USNR; HMC Quirino Galapon, USN;
Carl Berling; Edward P. Smith; Raymond S. Combs;
HM1 Gary Russell, USN; and HM1 Ronald F. Pettus, USN

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David P. McCormick, LT MC USNR; Edward P. Smith; Ronald F. Pettus
Quirino Galapon, HMC USN; Raymond S. Combs
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Fluorescent antibody reagents were prepared for the rapid diagnosis of acute respiratory disease caused by adenovirus in a military population. Methods are described for the preparation of high-titered antisera in rabbits, fractionation of globulins from the serum, conjugation with fluorescein isothiocyanate, and sorption to remove nonspecific staining. Fluorescein-tagged antisera were specific at dilutions of 1:20 to 1:30. (U)

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Virology Division
NAVAL MEDICAL FIELD RESEARCH LABORATORY
CAMP LEJEUNE, NORTH CAROLINA 28542

Bureau of Medicine and Surgery, Navy Department
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Submitted by:

WALTER E. BEAM, Jr
CAPT MSC USN
Chief, Virology Division

Approved by:

JESSE F. ADAMS
CAPT MC USN
Commanding Officer

SUMMARY PAGE

THE PROBLEM

Routine virus isolation and identification procedures require two to three weeks at a cost of up to $2.50 per test for materials alone. Furthermore, a rapid diagnosis of acute respiratory disease due to adenovirus in a military population would be useful in specific instances when antiviral chemotherapy is used, when there has been a rapid shift from one adenovirus type to another, or when the usual methods for virus isolation are not available.

FINDINGS

This laboratory has succeeded in preparing fluorescein-tagged adenovirus antisera for the rapid diagnosis of adenovirus disease in the field. Reagents are specific for adenovirus types 3, 4, and 7.

APPLICATION

The reagents will prove useful in epidemiological studies of adenovirus infection and in studies of the pathogenesis of adenovirus disease in the human host.

ADMINISTRATIVE INFORMATION

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ABSTRACT

Fluorescent antibody reagents were prepared for the rapid diagnosis of acute respiratory disease caused by adenovirus in a military population. Methods are described for the preparation of high-titered antisera in rabbits, fractionation of globulins from the serum, conjugation with fluorescein isothiocyanate, and sorption to remove nonspecific staining. Fluorescein-tagged antisera were specific at dilutions of 1:20 to 1:30.

INTRODUCTION

With the advent of live attenuated adenovirus vaccines[1] and the possibility of the use of chemotherapeutic agents[2] for viral infections, the rapid diagnosis of viral infections causing acute respiratory disease becomes ever more important. Fluorescent antibody procedures have been useful for the detection of adenovirus and *Herpesvirus hominis* antigens in infected cells.[3-7] We have turned our attention to the preparation of fluorescein-labelled antisera for staining epithelial cells scraped from the adenovirus-infected pharynx, or as an alternative, to identify virus-infected cells scraped from tissue culture tubes that show a cytopathogenic effect.

In this paper we describe our experience with the preparation and fluorescein-labelling of antisera, and we also describe a simplified technique for staining a variety of target antigens by the direct or indirect methods

METHODS

Tissues

Continuous human epithelium (HEp 2) cells were obtained from Microbiological Associates and were propagated in Eagle's minimum essential medium (MEM) supplemented with 2-5% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin added. HeLa-M cells were provided by the Department of Pediatrics, Ohio State University, Columbus, Ohio. Primary human embryonic kidney (HEK) cells were obtained from HEM Laboratories. All tissues except HeLa-M were mycoplasma free. Tissues were cultured periodically for detection of mycoplasma contamination

Virus Prototypes

Virus strains consisted of adenovirus type 3, strain G G ; type 4, strain RI 67; and type 7a, strain S-1058 *H. hominis* virus was strain Mayo 1814 All virus prototypes were free from mycoplasma contamination and were provided by Ms Sylvia Cunningham Medical Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md

Preparation of Antisera

Antisera were produced in white rabbits using complete or incomplete Freund's adjuvant (*vide infra*) Monolayers of HEp-2 cells in 32-oz Povitsky bottles were washed twice with Hank's balanced salt solution (BSS) to remove residual FCS, and inoculated with virus strains Cells and virus were maintained at 37°C in Eagle's MEM without FCS until 3+ to 4+ cytopathogenic effect (CPE) had developed Infected cells were removed by shaking or repeated freezing and thawing; antigens were pooled and stored at −70°C until animals were ready for inoculation

As an alternative, adenovirus antigens were prepared in HEK Tissue culture tubes (10 x 110 mm) containing primary HEK were washed twice in Hank's BSS and inoculated with adenovirus at high multiplicity Cells were maintained in serum-free Eagle's MEM At 3+ to 4+ CPE antigens were pooled, distributed in sterile vials and stored at −70°C

The immunization schedule used has been described.[8] Pre-immunization bleedings were obtained from the rabbits by cardiac puncture. The schedules were as follows: day 1, rabbits were inoculated subcutaneously with 4 0 ml undiluted adenovirus antigen emulsified in 4.0 ml complete or incomplete Freund's adjuvant (Difco); day 7, 1.0 ml of undiluted antigen intravenously and 5.0 ml of antigen intraperitoneally; day 14, 1.5 ml of antigen intravenously and 5.0 ml intraperitoneally; day 21, 2 0 ml of antigen intravenously and 5.0 ml intraperitoneally; day 28, trial bleeding A serum neutralization test was carried out on the trial bleeding serum. If antibody titers were satisfactory, 2 0 ml of additional antigen were given intravenously and animals were exsanguinated 10 to 12 days thereafter

H hominis antiserum was prepared as follows: after a pre-immunization bleeding, animals were inoculated subcutaneously with 2 0 ml of antigen emulsified in 2.0 ml incomplete Freund's adjuvant; on days 7, 14, and 21, 1 0 ml of antigen was given intravenously On day 28 a trial bleeding was performed. If the neutralizing antibody titer was sufficiently high, animals were sacrificed and sera were stored at −20°C

Serum Neutralization Tests

Rabbit hyperimmune sera to adenovirus types 3, 4, 7 and *H hominis* were tested for neutralizing antibodies in microtiter plates[9] (U shaped wells, Cooke Engineering) Virus strains at a dilution of 100 $TCID_{50}$ were incubated with antiserum dilutions 1 4 to 1:2048 for two hours at room temperature Antiserum-virus mixture (0 05 ml) in MEM supplemented with 5% FCS was

added to each well. Confluent monolayers of HEp 2 cells in 32-oz Povitsky bottles were trypsinized and 0.05 ml of a suspension containing 200,000 cells/ml was added to each well. Wells were overlayed with three drops of sterile mineral oil, and plates were incubated at 37°C for three days. Neutralizing antibody titers were defined as the highest dilution of serum which completely inhibited growth of virus after three days of incubation.

Serum Protein, Albumin, and Globulin Determinations

The Biuret technique was used. Test tubes were labeled B (blank), M (mixture), P (total protein), and A (albumin). One-half milliliter of standard, control, or test serum was added to each of the tubes labelled M, and 10 ml of 22.6% Na_2SO_4 solution were added to the tube labelled M. Two milliliters of the mixture in tube M were removed to tube P and 3.0 ml ethyl ether were added to tube M, shaken vigorously, and centrifuged at 1,000 rpm. After centrifugation, 2.0 ml of the clear bottom layer of tube M were removed to tube A. Four milliliters of Biuret reagent were added to all tubes marked B, P, and A, and those tubes were incubated at 37°C for 30 min. Optical densities (OD) were measured on a Beckman DB-G spectrophorometer at a wavelength of 550 mμ. Protein concentrations were calculated by the formula

$$\frac{\text{OD Unknown}}{\text{OD Standard}} \times \frac{\text{Concentration}}{\text{of Standard}} = \text{gm\% total protein}$$

Fractionation of Antisera

Globulins were prepared from the hyperimmune rabbit serum by precipitation with ammonium sulfate.[10] An equal volume of cold 3.52 M $(NH_4)_2SO_4$ was added dropwise to 5.0 ml of undiluted rabbit serum chilled in an ice bath. The mixture was incubated overnight at 4°C, and the precipitate was removed by centrifugation at 10,000 rpm for 30 min. The precipitate was resuspended in 2.5 ml of distilled water and was reprecipitated with 2.5 ml 3.52 M $(NH_4)_2SO_4$ solution. The twice-precipitated globulin was resuspended in 2.5 ml distilled water and dialized against 0.15 M sodium chloride for four days at 4°C. Total protein, albumin, and globulin were assayed by the methods described above.

Conjugation of Hyperimmune Globulin with Fluorescein[11]

Globulin suspended in 0.15 M sodium chloride was diluted with 0.5 M, pH 9.0, carbonate-bicarbonate buffer so as to contain 10 mg protein globulin per milliliter solution. Fluorescein isothiocyanate isomer I (FITC, BioQuest, Division of Becton, Dickinson and Company) was added to the globulin suspension so that the FITC/globulin ratio was 0.02 to 0.05 mg FITC per milligram of protein globulin. The mixture was incubated at 4°C for 18 hr and dialized against phosphate-buffered saline (PBS, pH 7.5) for five days or until the dialysate no longer showed fluorescence under a Wood's lamp. Conjugates were then centrifuged at 10,000 rpm for 30 min to remove particulate matter.

Rabbit Liver Homogenate

A fresh rabbit liver was removed, minced and homogenized in 0.15 M saline in a Waring blender.[11] Homogenized tissue was transferred to a large measuring cylinder and precipitated with five volumes of acetone. The supernate was removed by suction and was washed three times with saline. Additional lipids were removed by further washing with acetone. The tissue was transferred to a Buchner funnel, washed with acetone, dried overnight, ground on a mortar and passed through a fine sieve to remove fibrous particles. The resultant powder was resuspended in PBS and centrifuged. The supernatant was discarded, and the powder was stored at −20°C.

Sorption of Conjugates[12]

Sorption of fluorescent antibody conjugates was carried out with either HeLa M cells or rabbit liver homogenate to improve specific staining. Approximately twenty 32-oz Povitsky bottles of HeLa-M cells were required for sorption with 2.0 ml of conjugate. Cells for sorption were grown in Eagle's MEM supplemented with 2 to 5% FCS. When confluent monolayers had developed, cells were removed by incubation at 37° with 1.0% versene (EDTA). Cells were washed twice in PBS. One part washed, packed, HeLa M cells or rabbit liver powder was added to four parts of conjugate and shaken gently for two hours at room temperature and for 18 hr at 4°C. Cells and debris were removed by centrifugation at 20,000 rpm for one-half hour.

Carrier Slides for Fluorescent Staining

This laboratory used a technique borrowed from Dr. Werner Henle, Children's Hospital of Philadelphia. Four to six cover slips (6 x 30 mm, Corning) were affixed to 3 x1-inch carrier microscope slides as in Figure 1. Cover slip number, antigen, and conjugate were labelled on the tape. The tissue employed was coded by color for the tissue type, i.e., red for HeLa-M, and blue for HEp-2. Nail polish was used to help seal the junction between cover slip, tape, and microscope slide.

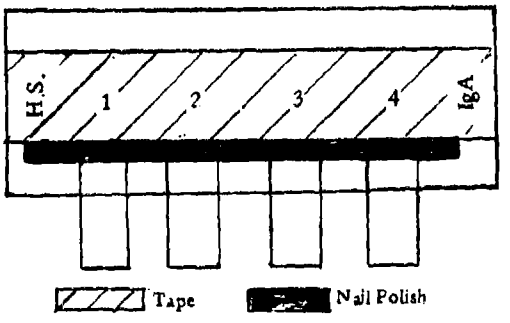

Fig. 1. Diagram of "Carrier Slide" with cover slips taped in place and ready to receive virus-infected cell suspensions. The tape was pre-labelled "H.S." to indicate *Herpesvirus hominis* target antigen and "IgA" to denote the use of anti-human IgA conjugated with FITC in that particular experiment. When staining was carried out, serial serum dilutions were added to cover slips 1, 2, 3, 4. After the antigens were stained, the cover slips were broken from the carrier slide and mounted cell-side-down on a pre-labelled microscope slide for fluorescent microscopy.

Target Antigens

Target viral antigens for fluorescent antibody staining by the direct or indirect technique were prepared the same as virus for rabbit immunization except that HeLa-M cells were used exclusively. When 4+ CPE developed, cells were removed to a centrifuge tube, washed three times in PBS, and resuspended in approximately 10 ml PBS. Two drops of the infected cell suspension were placed on a cover slip (*vide supra*) and examined for cell concentration under the inverted microscope. Cells were then diluted in PBS or concentrated by centrifugation so that the cells on the cover slips just touched each other at the edges. Cover slips were dried at 37°C and stored at 4°C until staining was carried out. Fixation of infected cells in acetone or alcohol was not necessary. Best results were obtained when infected target antigens were stained the same day they were harvested from the Povitsky bottles.

Conjugate Titrations

Ten-fold dilutions of conjugate 1:10 through 1:320 were prepared. Two drops of each conjugate dilution were placed on separate cover slips containing the appropriate antigen, were incubated in a humid chamber at 37°C for 45 min, and were washed in Coplin jars twice for 15 min in PBS. Excess salt was removed by washing for 5 min in distilled water, pH adjusted to 7.2 with a concentrated bicarbonate solution. The stained cover slips were permitted to dry. Both infected and uninfected cells were used as target antigen, and a conjugate specific for a different antigen was used as a negative control.

Blocking studies were carried out to verify the specificity of the conjugates. Unconjugated rabbit antiserum containing a high titer of specific antibody to herpes or adenovirus was applied to the appropriate cover slips at a dilution of 1:4. After incubating for 45 min in a humidified atmosphere, the slips were washed twice for 15 min each in PBS and then stained with fluoresceinated globulin as described above.

Mounting Fluid

A clear fluid which does not absorb or emit ultraviolet light was used. We dissolved 10 gm Elvanol (Du Pont) grade 71-30 by heating in 80 ml PBS. Forty milliliters of glycerol were then added and the mixture was autoclaved at 10 lb for 10 min and dispensed in 4-dram vials. Mounting fluid was warmed in a water bath to liquify it before use.

Fluorescence Microscopy

Stained and unstained target antigens were observed through a Richart Zetopan research microscope. Light originated from an HBO L-2 super pressure mercury vapor lamp (Osram), passed through a 3.0 mm BG 12 exciter filter, through the specimen, and finally through a GG 9 or GG 13 barrier filter. A dark field condenser was used, and photographs were taken of the stained cells under glycerin immersion.

RESULTS AND DISCUSSION

Both HEp 2 and HEK may be used for producing immunizing antigens, although adenovirus grown in HEK would appear to give higher neutralizing antibody titers, especially when complete Freund's adjuvant is used (Table 1). Adenovirus neutralizing antibody titers of 1:512 were obtained when adenovirus types 3 and 4 were prepared in HEp 2 cells and used for immunizing rabbits by the schedule described in the methods. The antisera were type specific, and there were no cross-reacting neutralizing antibodies.

We fractionated hyperimmune antisera by several methods including precipitation with saturated ammonium sulfate solution and agar block electrophoresis. However, the method of Cherry[10] in which sera were twice precipitated with an equal volume of 3.52 M $(NH_4)_2SO_4$ gave a high yield of globulin which was contaminated by albumin at a concentration of only 1.1% after the second precipitation. A low albumin concentration prevents non-specific staining because albumin has a much higher affinity for FITC than do globulins.

Adenovirus conjugates that had not been subjected to sorption demonstrated non-specific staining against the battery of target antigens at dilutions of up to 1:20 (Table 2). However, one sorption with packed HeLa M cells removed the heterospecific reactivity. It is possible that the rabbits may have been immunized with small amounts of calf serum, even though cell monolayers were washed prior to inoculation of virus, and this component may have been removed by the sorption procedure. *H. hominis* conjugate gave a high titer of specific antibody after sorption with both rabbit liver and HeLa M cells.

TABLE 2

Reduction in Non-specific Staining by Sorption with HeLa M Cells. Adenovirus Type 7 Conjugate is Titered Against the Battery of Antigens

Dilution of Conjugate	Antigen		
	Ad. 3	Ad. 4	Ad. 7
Before Sorption			
1:10	+	+	+
1:20	-	+	+
1:40	-	-	-
1:80	-	-	-
1:160	-	-	-
After Sorption			
1:10	-	-	+
1:20	-	-	+
1:40	-	-	-
1:80	-	-	-
1:160	-	-	-

TABLE 1

Titers of Antisera Produced in Rabbits by Immunization with Adenovirus Types 3, 4, 7 and *Herpesvirus hominis*

Rabbit Antiserum To	Freund's Adjuvant*	Grown In	Neutralizing Antibody Titer Virus Type			
			3	4	7	*H. hominis*
Adenovirus type 3	I	HEp 2	>1:512	<1:4	<1:4	<1:4
Adenovirus type 4	I	HEp 2	<1:4	>1:512	<1:4	<1:4
Adenovirus type 7	I	HEp 2	<1:4	<1:4	1:64	<1:4
Adenovirus type 7	C	HEK	<1:4	<1:4	1:2048	<1:4
Herpesvirus hominis	I	HEp 2	<1:4	<1:4	<1:4	1:128

*I = Incomplete Freund's adjuvant.
C = Complete Freund's adjuvant.

The conjugates which we prepared met all the accepted criteria for specificity. In addition to the specific staining which has already been described, unconjugated specific antisera blocked the binding of conjugate to the target antigen (Table 3). Conjugates did not stain uninfected cells, and antisera conjugated with FITC did not stain heterospecific antigens.

TABLE 3

Blocking Effect of Specific Antibody When Incubated with Target Antigen Prior to Application of *Herpesvirus hominis* Conjugate

Dilution of Conjugate	No Blocking Antibody	Blocking Antibody
1:20	+	+
1:40	+	-
1:80	+	-
1:160	+	-
1:320	-	-

We found dialysis far superior to column chromatography as a method for removing unconjugated FITC from the FITC-globulin reaction mixtures. As an alternative to dialysis, we attempted to purify conjugates by passing them through a column of Sephadex G-50. Good separation of the unbound FITC was achieved; however, conjugates recovered from the column did not stain specifically at high dilutions, even when concentrated five- to ten-fold.

The carrier slide technique illustrated in Figure 1 was most useful. Only small quantities of reagents were required, color-coding of carrier slides for a specific cell type reduced laboratory error, and washing was simplified by suspending the slides with attached cover slips directly in Coplin jars. The Virology Division of this laboratory has used this technique for a wide variety of both direct and indirect staining procedures including the detection of antibody to Epstein-Barr virus, studies of human antibody responses to colonization by meningococcus types B, C, and Y, and analysis of IgM, IgA, and IgG-specific herpes antibody in chronic *Herpesvirus hominis* excretors. Because it is inexpensive and easy to use, the method is particularly useful in the field laboratory which does not have access to automated fluorescence staining equipment.

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